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Interactions of arsenic, copper, and zinc in soil-plant system: Partition, uptake and phytotoxicity

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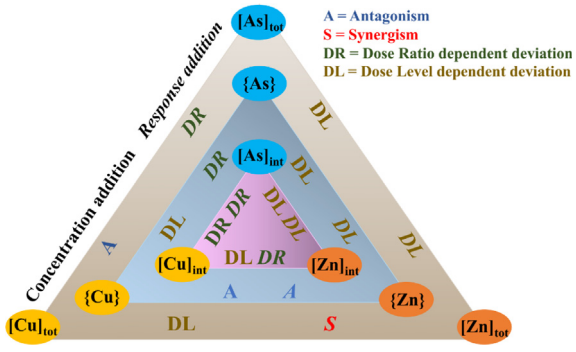
HIGHLIGHTS

- K_d of one metal(loid) was most often inhibited by the presence of the other one.
- The uptake of As, Cu, or Zn was decreased or unaffected by adding the second metal.
- Observed interaction patterns for binary mixtures depending on the model applied
- CA and RA models suggest additive effects for the ternary mixtures (As-Cu-Zn).

GRAPHICAL ABSTRACT



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ABSTRACT

Arsenic, copper, and zinc are common elements found in contaminated soils but little is known about their combined effects on plants when presented simultaneously. Here, we systematically investigated the phytotoxicity and uptake of binary and ternary mixtures of As, Cu, and Zn in a soil-plant system, using wheat (*Triticum aestivum*) as model species. The reference models of concentration addition (CA) and response addition (RA) coupled with different expressions of exposure (total concentrations in soil ($[M]_{tot}$, mg/kg), free ion activities in soil solution ({M}, μM), and internal concentrations in plant roots ($[M]_{int}$, μg/g)), were selected to assess the interaction mechanisms of binary mixtures of As—Cu, As—Zn, and Cu—Zn. Metal(loid) interactions in soil were estimated in terms of solution-solid partitioning, root uptake, and root elongation effects. The partitioning of one metal(loid) between the soil solution and solid phase was most often inhibited by the presence of the other metal(loid). In terms of uptake, inhibitory effects and no effects were observed in the mixtures of As, Cu, and Zn, depending on the mixture combinations and the dose metrics used. In terms of toxicity, simple (antagonistic or synergistic) and more complex (dose ratio-dependent or dose level-dependent) interaction patterns of binary mixtures occurred, depending on the dose metrics selected and the reference models used. For ternary mixtures (As-Cu-Zn), nearly additive effects were observed irrespective of dose descriptors and reference models. The observed interactions in this study may help to understand and predict the joint toxicity of metal (loid)s mixtures in soil-plant system. Mixture interactions and bioavailability should be incorporated into the regulatory framework for accurate risk assessment of multimetal-contaminated sites.

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1. Introduction

Arsenic (As) is a known carcinogen and mutagen as well as non-essential trace element for organisms (Abbas et al., 2018). Copper (Cu) and zinc (Zn) are essential micronutrients for plants and animals, but may exert toxic effects at high concentrations (Guzman-Rangel et al., 2018; Kader et al., 2018). The ranges of geochemical background levels for As, Cu, and Zn are 0.05–666, 0.3–395, and 2.8–1396 mg/kg, respectively (Reimann et al., 2018). The processing of the pyrometallurgical production is causing a large release of metal(loid)s such as As, Cu, and Zn simultaneously into the soil environment (Gore et al., 2007). The broad usage of chromate-copper-arsenate together with zinc-sulphate in wood preservative may also trigger co-contamination of As with Cu and/or Zn (Kader et al., 2017). Given the fact that As is a non-essential metal(loid) and Cu/Zn is essential, the toxicity of one metal(loid) may be alleviated or aggravated in the presence of the other. Therefore, there is a need for understanding the mutual interactions of mixtures of As, Cu, and Zn as well as their effects on organisms.

Most contaminated soils contain elevated concentrations of several toxic metals, and the negative environmental effects of their combination are different to those caused by each metal individually (Gopalapillai and Hale, 2017). In this regard, experiments to assess the phytotoxic effects of metals are more realistic if they are undertaken with such mixtures (Nys et al., 2017). However, it is impractical to evaluate all possible mixture combinations. Conceptual models were therefore developed by simply adding doses (i.e., concentration addition, CA) and responses (i.e., response addition, RA) to predict mixture effects based on the assumption that mixture components do not impact each other's toxicological action (Altenburger et al., 2004). According to the mathematical connotation of both concepts (CA or RA), they can be easily applied to all mixtures without considering the modes of action (MOAs) of the components in the mixture. Different metals can have different uptake mechanisms, or can compete with different ions (e.g. Ca^{2+} or Na^{+}) for uptake at their carrier systems (Niyogi and Wood, 2004), suggesting that the RA concept should be used. However, inside biota different metals can have equal modes of action, for example by causing oxidative stress, or being detoxified by shared mechanisms like metallothioneins or phytochelatins (Briat and Lebrun, 1999), suggesting that the CA concept should be used. If the mixtures induce a less (more) severe effect as determined by either of these reference models, interactions are defined as antagonistic (synergistic) (Loureiro et al., 2010). These mutual interactions can be further quantified by introducing extra parameters using the MIXTOX model framework on the basis of CA and RA models (Jonker et al., 2005). To our knowledge, no detailed studies have been published on quantitative modeling of mixture toxicity involving anionic metal(loid)s in soil.

Typically, mixture interactions are complex to study and no general pattern in the observations is reported in soil because components in mixtures may interact at different levels: within the soil, during the uptake processes (Qiu et al., 2016), and on the target sites in the cell (Gopalapillai and Hale, 2017). Distinguishing interactions at different levels will help to understand different interaction patterns (antagonistic, synergistic, and additive) in soil. The difference between interaction patterns may result from the selected model species and dose metrics. To date, not many phytotoxicity studies on metal(loid) mixtures are available in soil systems, and most of them are analyzed on the basis of total soil concentrations (Das et al., 2016; Kader et al., 2017; Sanchary and Huq, 2017). For example, the study of Cao et al. (2007) observed antagonistic interactions between As and Cd on root elongation of wheat (*Triticum aestivum* L.) based on total metal concentrations in soil. Egodawatta et al. (2019) identified a synergistic interaction of As—Sb to shoot dry mass and additive toxicity to shoot length of spinach (*Ipomoea aquatica*) using total soil concentrations. It should be noted that interactions related to bioavailability should not be ignored, especially in soil. Kader et al. (2018) focused on pore-water concentrations to determine interactions between As and Cu mixtures. They observed an additive response on phytotoxicity to cucumber (*Cucumis sativus* L.) exposed to As—Cu mixtures when using pore-water concentrations but not for total concentrations. Compared with external concentrations (total soil concentration or pore-water concentration), internal concentration allows integrating overall interactions of the metals occurring at exposure and uptake level. Wyatt et al. (2016) observed complex interaction patterns between Se and Hg to nematodes (*Caenorhabditis elegans*), including antagonistic, synergistic and additive interactions based on concentrations in the test medium; whereas no antagonistic interaction was found on the basis of internal concentrations of Se/Hg. Additionally, the use of CA or RA models may display consistent or inconsistent interaction patterns, while these interactions changed with soil types (Iwasaki and Gauthier, 2016). For example, mixture analyses based on either the CA or RA model found significant antagonism between As and Zn in two soils with total soil concentrations as the dose metric, whereas additive effects were observed in one soil with the lowest content of Fe-oxides (Guzman-Rangel et al., 2018). Consequently, in order to identify where mixture interactions of metal(loid)s occur and what is their magnitude, it is necessary to determine and compare them using both CA and RA models on the basis of external and internal concentrations.

This study aimed at analyzing and modeling the phytotoxicity and uptake of As, Cu, Zn and their mixtures in a soil-plant system, and to shed light on the interaction mechanisms of metal(loid)s mixtures, taking bioavailability into account. To achieve this aim, the observed toxicity and uptake of metal mixtures to wheat (*Triticum aestivum* L.) was related to total concentrations in soil, free ion activities in soil solution, and internal concentrations in plant roots.

2. Material and methods

2.1. Test substrates

The natural standard soil LUFA 2.2 was purchased from LUFA Speyer, Germany (Table S1). This soil has been recommended and used in many terrestrial ecotoxicity tests (Waalewijn-Kool et al., 2013; Zhang and Van Gestel, 2017). The LUFA 2.2 soil was spiked with either NaH_2AsO_4 (>99%, Sigma-Aldrich), $CuCl_2$ (>98%, Merck), or $ZnCl_2$ (>98%, Merck) to obtain three different stock soils with nominal concentrations of 1000 mg As/kg dry soil, 1000 mg Cu/kg dry soil and 2000 mg Zn/kg dry soil, respectively. The spiked soils were moistened to reach field water capacity and equilibrated at 25 °C for two months. After that, the stock soils were leached with artificial rainwater to prevent excessive salt stress (Smolders et al., 2015). The single-metal (As, Cu, and Zn) and mixture (As—Cu, As—Zn, Cu—Zn, and As-Cu-Zn) treatments were prepared by mixing certain amounts of leached stock soils with the non-spiked soils (see Table S2 for detailed mixture combinations). These concentrations were designed to produce entire dose response curves based on the preliminary range-finding test. Control soils received water only. Soil pH (0.01 M $CaCl_2$) of all treatments was adjusted to 6.0 ± 0.5 by adding calcium oxide. Before use, these soil treatments were equilibrated at 25 °C in a climate room for another two months.

2.2. Toxicity tests

Root elongation tests with wheat (*Triticum aestivum* L.) were performed following an ISO standardized protocol (ISO 11269-1: 2012) with slight modifications (Qiu and Smolders, 2017). Three germinated wheat seeds with initial root length of 1–2 mm were introduced into each plastic pot (115 mm in length × 30 mm in diameter) fully filled with test soil. Three replicates were used for each treatment and control. Test pots were randomly placed in a growth chamber (20 °C, 75% relative humidity, photoperiod cycle of 16 h light-8 h dark, and photon flux density of 450 μmol/m^2·s). Water loss in the test pots was checked daily and replenished by adding deionized water. After exposure for 5 days, plants were gently taken from the soils and the length of the

longest root of each seedling was measured. The 5d root elongation test is an acute test, which can provide rapid early warning of soil toxicity. The entire roots were washed with deionized water to remove soil particles and then submerged in 0.02 M Na_2EDTA solution for 15 min to remove surface-bound metals (De Schamphelaere et al., 2005). After that, the roots were flushed with deionized water and dried at 60 °C for further analysis.

2.3. Chemical analysis and speciation calculations

Soil pH was measured in 0.01 M $CaCl_2$ extract using a pH meter (Metrohm AG). Soil texture, organic matter content, maximum water holding capacity, and cation exchange capacity were determined following routine analytical methods (see notes to Table S1 for details) (Hamels et al., 2014). At the end of the toxicity tests, a soil subsample from each treatment was dried at 60 °C in the oven. Dried soils and wheat roots were digested with *aqua regia* for metal analyses. *Aqua regia* was freshly prepared by mixing concentrated HCl (36.5–38.0%) and concentrated HNO_3 (69.0–70.0%) with a ratio of 3:1. For porewater collection, the soils were sampled immediately after removing the wheat roots. These fresh soil samples were subjected to double-chamber centrifugation (15 min at 3000*g*) and subsequent filtration over a 0.45 μM membrane filter (Qiu et al., 2016). Total metal concentrations in soil, dissolved concentrations of elements (As, Cu, Zn, Na, Mg, K, and Ca) in soil pore water, and internal metal concentrations in plant roots were measured by ICP-OES (Perklin Elmer 3300 DV). Quality control of metal analysis was performed by including certified reference materials (ISE 989 for soils and NIST 1573a for plants). Recoveries of metals were 92% to 108% of the certified values. Major anions (Cl^-, NO_3^-, and SO_4^{2-}) were determined by Dionex (DX500). Dissolved organic carbon (DOC) was measured using a TOC analyzer (Multi N/C 2100 s).

Speciation of As, Cu, Zn and major cations (Ca, Mg, Na, K) in the soil solutions were calculated by the chemical speciation model WHAM VII (Tipping et al., 2011), using measured total elemental concentrations, anion concentrations, pH, temperature (293 K), pCO_2 ($10^{-2.5}$ atm) and colloidal fulvic acid (FA) as input variables. The FA was calculated from DOC by assuming that dissolved organic matter contained 50% C (DOM = 2 DOC) and that 65% of DOC was active as fulvic acid. Since Fe^{3+} and Al^{3+} can compete strongly with other cations for binding to FA, their activities were included in the speciation calculation as well. Free Fe^{3+} and Al^{3+} activities were estimated from solubility products of $Fe(OH)_3$ (log K_{solu} = 2.7) and $Al(OH)_3$ (log K_{solu} = 8.5) (Tipping et al., 2003). The activities of As in the present study were the sum of $HAsO_4^{2+}$ and $H_2AsO_4^-$ ion activities because $H_2AsO_4^-$ (90.6%) and $HAsO_4^{2-}$ (9.36%) were the dominant species at pH 6.0 (Wang et al., 2008).

2.4. Data analysis and mixture modeling

A logistic dose−response model was used to analyze the toxicity data of single metal exposures:

$$\mathrm{RRE}(\%) = \frac{100}{1 + \left(\frac{c_i}{\mathrm{EC50}}\right)^{\beta_i}} \quad (1)$$

where RRE is relative root elongation (root length relative to the length in the control soil, %), *c* is the exposure dose (i.e., total metal concentration in soil ($[M]_{tot}$, mg/kg), free metal ion activity in soil porewater ({M}, μM), and internal metal concentration in plant roots ($[M]_{int}$, μg/g)), EC50 is the effective concentration triggering 50% effect, β is the slope parameter.

Toxicity of binary and ternary metal mixtures was assessed with response addition (RA) and concentration addition (CA) model, respectively. When applying the RA model, mixture components are assumed to exhibit different modes of action (Iwasaki and Gauthier, 2016). The mixture effects (RRE_{mix}) were predicted from the joint probability of statistically independent events:

$$\mathrm{RRE_{mix}}(\%) = \prod_{i=1}^{n} \mathrm{RRE_i} \quad (2)$$

where RRE_i is the predicted relative root elongation when exposed to single metals (As, Cu, or Zn).

For binary (As—Cu) or ternary (As-Cu-Zn) mixtures,

$$\mathrm{RRE_{mix}}(\%) = \frac{\mathrm{RRE_{As}} \times \mathrm{RRE_{Cu}}}{100} \text{ or } \frac{\mathrm{RRE_{As}} \times \mathrm{RRE_{Cu}} \times \mathrm{RRE_{Zn}}}{100^2} \quad (3)$$

When applying the CA model, mixture components were assumed to have similar mode of action (Jonker et al., 2005).

$$\sum_{i=1}^{n} \frac{c_i}{\mathrm{EC50_i} \times \left(\frac{100-\mathrm{RRE_{mix}}}{\mathrm{RRE_{mix}}}\right)^{1/\beta_i}} = 1 \quad (4)$$

where $EC50_i$ and β_i are the dose-response parameters for each metal when applied individually, c_i is the concentration of each mixture component. For ternary mixtures of As, Cu and Zn,

$$\frac{c_{As}}{\mathrm{EC50_{As}} \times \left(\frac{100-\mathrm{RRE_{mix}}}{\mathrm{RRE_{mix}}}\right)^{1/\beta_{As}}} + \frac{c_{Cu}}{\mathrm{EC50_{Cu}} \times \left(\frac{100-\mathrm{RRE_{mix}}}{\mathrm{RRE_{mix}}}\right)^{1/\beta_{Cu}}} + \frac{c_{Zn}}{\mathrm{EC50_{Zn}} \times \left(\frac{100-\mathrm{RRE_{mix}}}{\mathrm{RRE_{mix}}}\right)^{1/\beta_{Zn}}} = 1 \quad (5)$$

To find the CA-predicted RRE_{mix}, Eqs. (4) and (5) were numerically solved using the generalized-reduced-gradient-iterative solver function (JMP 16.0, SAS Institute).

The potential interactive effects including antagonistic, synergistic, dose-ratio dependent (DR), and dose-level dependent (DL) deviations were evaluated following a more elaborate MIXTOX framework (Jonker et al., 2005). This framework uses the CA or RA reference models on the basis of the whole data set. Deviation from additivity could be quantified by modifying the original models with a parametric function (*G*). The deviation function is related to the expression of the mixture components,

$$\mathrm{q}_i = \frac{1}{1 + \left(\frac{c_i}{\mathrm{EC50}}\right)^{\beta_i}} \text{ for the RA model} \quad (6)$$

$$\mathrm{z}_i = \frac{\mathrm{TU50}_i}{\sum \mathrm{TU50}} \text{ for the CA model} \quad (7)$$

where q_i represents individual effects of *i* in the mixture; z_i represents the relative toxic units (TU) of *i* in the mixture.

An additional parameter (*a*) is introduced into this deviation function to test for significant synergistic/antagonistic deviations from the reference model. Moreover, the MIXTOX analysis allows for identifying DR and DL deviation patterns by further adding extra parameters using a stepwise approach. Specifically, this method consists of three steps (Asselman et al., 2013). In the first step, a reference model is fitted to the single toxicity data and is then used to predict toxicity in the mixture treatments. A large difference between predicted and actual responses is an indication of interactions. In the next step, 2 nested models are fitted to all data: the reference model (CA or RA) and the reference model extended with a deviation function using an additional parameter *a*. In the third step, the two models are compared using an *F*-test. If the extended model performs significantly better than the reference model, significant interactions are detected. An *a*-value larger than 0 indicates antagonistic interactions, an *a*-value smaller than 0 indicates synergistic interactions. This analysis is performed with both the CA and RA reference models based on different exposure metrics (i.e., $[M]_{tot}$, {M}, and $[M]_{int}$). The explanation of the extra parameters is shown in Table S3.

3. Results and discussion

3.1. Soil properties

The properties of the standard soil LUFA 2.2 are presented in Table S1. Soil pH (in 0.01 M $CaCl_2$) was acidic (5.45). The clay content, organic matter (OM) content, and cation exchange capacity (CEC) were 7.7%, 4.35%, and 8.24 $cmol_c$/kg, respectively. The total As, Cu, and Zn concentrations in the soil were 7.0, 16.9, and 78.4 mg/kg dry soil, respectively (Table S1). Main soil properties should not show strong differences from the control after spiking treatments and only the metal (loid) contents and the pH were determined (Table S2).

3.2. Solution-solid partitioning of As, Cu, and Zn

The interactions of binary mixtures of As—Cu, As—Zn, and Cu—Zn at the exposure levels tested were investigated by directly comparing the partition coefficients (K_d) of each metal in exposures to single metals and binary combinations. The K_d is defined as the ratio of the total concentrations in the soil and in the pore water (Sauvé et al., 2000), and indicates partitioning of the metal between the solid and solution phases. Partition coefficients of As, Cu, and Zn when spiked singly and in mixtures are presented in Fig. 1. Interestingly, there are opposite trends of K_d values to increasing soil concentrations. For single elements, larger concentrations of Cu in soil benefited its relative adsorption as shown by the increasing K_d values. In contrast, higher concentrations of As or Zn decreased the K_d values. In most situations, saturation of potential adsorption sites will occur, leading to a decreased proportion being sorbed at higher total metal concentrations (Sauvé et al., 2003) resulting in lower K_d values (Sauvé et al., 2000).

In the mixtures, partitioning of As increased drastically in the presence of a fixed amount of total Zn (550 mg/kg) compared with that in the treatments of As alone while no significant changes in the partitioning of As were observed in the presence of Cu (mean value of 330 mg Cu/kg dry soil). Partitioning of Cu decreased drastically with the addition of a fixed amount of total As (mean value of 60 mg As/kg dry soil) while a similar decline was only observed for Zn addition when the total Cu concentration was >102 mg/kg (with Zn fixed at mean value of 550 mg Zn/kg dry soil). Partitioning of Zn was reduced in the presence of either As (60 mg/kg) or Cu (330 mg/kg) when total Zn concentration was below ~1000 mg/kg, while no significant changes were found above that level. Arsenic likely precipitated due to its very low solubility product (K_{sp}) resulting in a decreased concentration in the soil pore water. In addition, the presence of organic matter and of Fe, Mn, and Al oxides could have significant impacts on the partitioning of As in soils. The association of As with the Fe—As and Al—As fractions changed greatly, especially in metal-spiked soils (Fayiga et al., 2007). The effects of As—Zn interaction could be explained by the formation of less soluble Zn-arsenate complexes in the soil (Guzman-Rangel et al., 2017). Similarly, a reduction of As porewater concentration in the presence of Zn was found by Kader et al. (2017). An increase of As adsorption on magnetite in the presence of Zn was noted by Yang et al. (2010). For As—Cu, potential formation of As—Cu surface precipitates might depend on the ratio of As and Cu in the soil solution (Kader et al., 2018). The comparison among K_d values of Cu or Zn in single exposures and in mixtures suggests that the mutual interactions of Cu and Zn were mainly antagonistic and due to competition of Cu and Zn for sorption onto remaining binding sites in soil (Qiu et al., 2016).

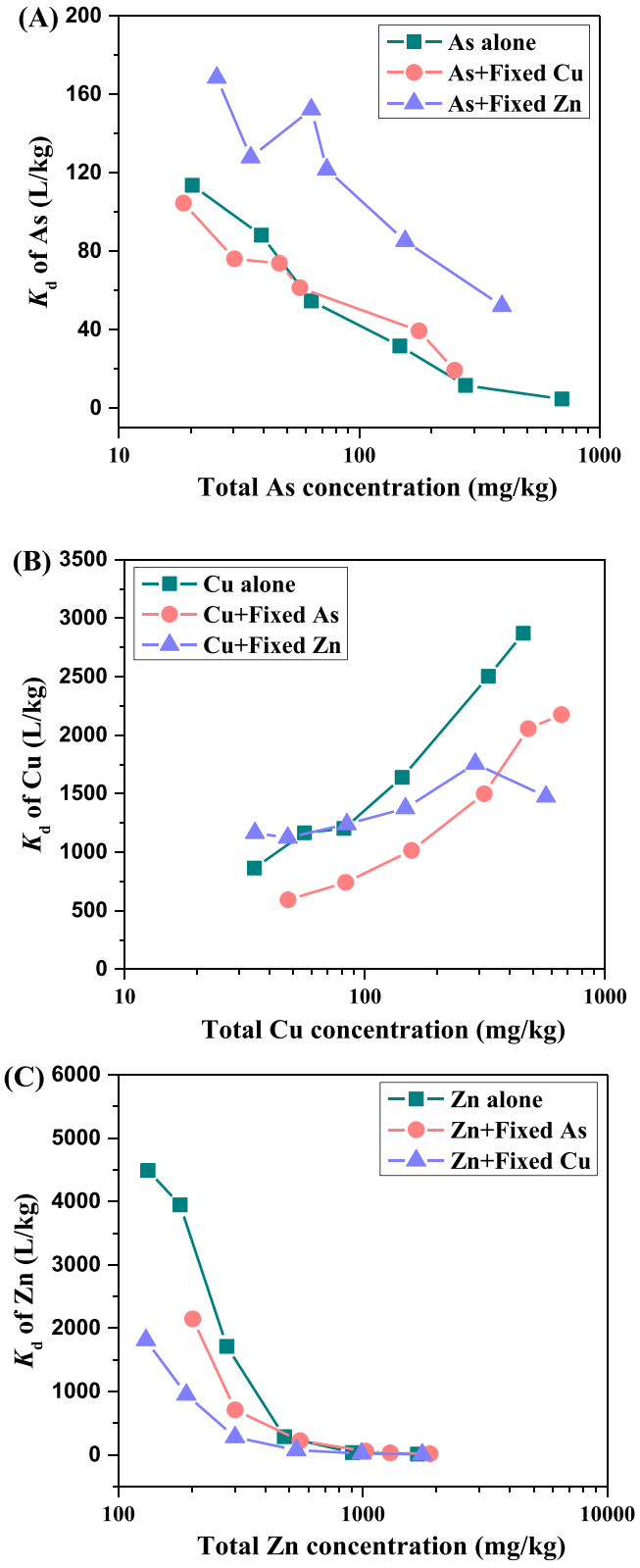

Fig. 1. Partition coefficients (K_d) of As, Cu, and Zn in LUFA 2.2 soil when present singly and in mixtures. In binary mixtures, the concentration of As, Cu, and Zn was fixed at 60, 330, and 550 mg/kg, respectively. Solid lines schematically show the trends.

3.3. Toxicity of single As, Cu, and Zn

Among the three elements tested, As was the most toxic when dose was expressed as $[M]_{tot}$. Toxicity of As was about 3-fold higher than that of Cu and 9-fold higher than that of Zn (Table 1). In contrast, Cu was the most toxic when dose was expressed as {M} or $[M]_{int}$. Zinc was the least toxic element whichever one of three dose descriptors was used (Table 1). These results are consistent with phytotoxicity results reported previously (Kader et al., 2017, 2018; Qiu et al., 2016).

Table 1
Single metal dose-response parameters for the toxicity of As, Cu and Zn applied singly to wheat root elongation in LUFA 2.2 soil, using total soil metal concentrations ($[M]_{tot}$, mg/kg), free metal ion activity in soil solution ({M}, μM), and internal metal concentrations in wheat roots ($[M]_{int}$, μg/g) as dose descriptors, respectively. The standard errors are listed in brackets. EC50 is the concentration or free ion activity at which root elongation was reduced by 50% compared to the untreated control, β is the slope of the dose-response relationship.

	Total soil concentrations			Free ion activity			Internal concentrations		
	EC50$[M]_{tot}$ (mg/kg)	β	R^2	EC50{M} (μM)	β	R^2	EC50$[M]_{int}$ (μg/g)	β	R^2
As	115 (17)	1.99 (0.49)	0.95	34.2 (9.12)	1.07 (0.26)	0.95	359 (61)	1.65 (0.41)	0.94
Cu	376 (10)	7.01 (1.15)	0.98	0.01 (0.003)	2.00 (0.73)	0.85	139 (3)	13.2 (2.59)	0.95
Zn	996 (69)	4.59 (1.56)	0.96	207 (36.2)	2.27 (1.16)	0.95	1608 (177)	2.68 (0.75)	0.97

3.4. Root uptake of As, Cu, and Zn

Concentrations of As, Cu, and Zn measured in the plant roots are plotted against the corresponding $[M]_{tot}$ and {M} (Fig. 2). Root As concentrations were reduced in the presence of a fixed amount of either Cu (330 mg/kg) or Zn (550 mg/kg) when $[M]_{tot}$ was used as the dose descriptor (Fig. 2A). A significant decrease of As uptake in cucumber shoots with the addition of Cu based on either total concentrations in soil or measured pore-water concentrations was also noted by Kader et al. (2018). Similarly, As uptake in the Chinese ladder fern *Pteris vittata* L. was reduced in the presence of Cd, Ni, Pb and Zn when total soil concentrations were lower than 200 mg/kg (Fayiga et al., 2004). Root As concentrations in the ladder fern plants reduced with an increase in metal concentration following the order Ni < Cd < Zn < Pb (Fayiga et al., 2007). In contrast, the uptake of As by the plant *Silene vulgaris* was not affected by Cd and the interaction between As and Cd was concentration-dependent (Sneller et al., 2000). In our study, the reduction in root uptake of As in the presence of Cu or Zn indicated antagonistic interactions on the basis of $[M]_{tot}$. This difference disappeared when {M} was used as the dose descriptor (Fig. 1D). In other words, root As concentrations were determined by free As (sum of $HAsO_4^{2-}$and $H_2AsO_4^-$) ion activity. The observed antagonistic influence of Cu/Zn on As uptake disappeared when using {M} as the dose descriptor.

When related to either $[M]_{tot}$ or {M}, the addition of a fixed amount of As (mean value of 60 mg As/kg dry soil) drastically decreased root Cu concentrations while no significant changes in root Zn concentrations were observed when As was added (Fig. 2B, C, E, and F). Root metal concentrations at the same free metal ion activity differed between the single and mixture treatments for Cu but not for Zn, suggesting that Cu uptake was not solely determined by Cu^{2+} and that Zn uptake was dominated by Zn^{2+}. Our results showed that As had an antagonistic effect on Cu uptake but no significant effect on Zn uptake irrespective of the dose descriptors. However, Cu uptake was not significantly affected by As in *Cucumis sativus* L. based on pore-water concentrations (Kader et al., 2018). The uptake mechanisms of As and Zn are quite different, which may be the reason why As had no effect on Zn uptake in the present study. Another research has reported that the presence of As resulted in a significant decrease of Zn uptake ($p < 0.05$) using pore-water concentrations (Kader et al., 2017). As a consequence, the overall metal(loid) uptake by plants might be influenced by solubility, soil chemistry and uptake mechanisms. In addition, cationic metals (Cu and Zn) and anionic metal(loid)s (As) exist in solution as ions with an inverse charge and they may affect each other's uptake by plants by interacting on the root surface (Smith et al., 2001). Cao et al. (2007) reported that As and Cd showed a synergistic interaction in solution culture. The authors proposed that the total charge of the plasma membrane surface varied from negative to neutral in the presence of positively charged Cd, which could be easily adsorbed to the root surface, and thus the concentration of the anionic metal (loid) As would increase at the root surface resulting in more chance of being taken up by the plant roots.

3.5. Modeling mixture interactions and toxicity of As—Cu, As—Zn, Cu—Zn, and As-Cu-Zn

To unravel the role of interactions occurring at different levels in soil during exposure to the binary mixtures of As—Cu, As—Zn, Cu—Zn and the ternary mixtures of As, Cu, and Zn, the observed toxic effects were compared with values predicted by the reference models when expressed on the basis of different dose descriptors ($[M]_{tot}$, {M}, or $[M]_{int}$) (Figs. 3 and 4). Based on the dose-response parameters obtained from the single metal(loid) exposures, CA or RA displayed similar prediction precisions in estimating mixture toxicity of As—Cu, As—Zn, and Cu—Zn (Table S4). In general, the use of $[M]_{tot}$ coupled with conventional toxicity models is not sufficient to normalize toxicity variations among soils without considering bioavailability (Qiu and He, 2017). Available metal concentration is therefore accepted as a more reliable indicator of its phytotoxicity than total metal concentration in soil (Qiu et al., 2016). Which expression of exposure to metals in soil is the best descriptor of the potentially bioavailable metal concentration is, however, inconsistent in the ecotoxicological literature. Given the assumption that soil pore water is the dominant route of exposure for plants, some authors supposed that the free metal ion in soil solution is the main toxic species for plants (Thakali et al., 2006). Other researchers consider the concentration accumulated in organisms a better indicator than external concentrations for predicting metal toxicity (He et al., 2015). In the present study, no good correlation was observed using $[M]_{int}$ as the expression of exposure in all the tested binary mixtures, irrespective of the used reference models (Table S4). This might result from the completely different sites determining uptake and toxicity of metals, where the site of toxic action is within the wheat roots rather than on the surface (Borgmann et al., 2008).

The global mixture interactions of As—Cu, As—Zn, and Cu—Zn were further quantified using the MIXTOX framework. Results of the binary mixtures (As—Cu, As—Zn, and Cu—Zn) relative to the RA model are described in the Supporting Information (SI) Text S1. The output parameters of the MIXTOX model and the identified interaction patterns are summarized in Table 2. When we related toxicity to $[M]_{tot}$, with inclusion of the parameter a in the CA model to indicate antagonism or synergism, the sum of the square residuals (SS) was reduced significantly ($p(\chi^2) < 0.01$) in all binary mixtures (Table 2). Significant antagonistic interactions were found for the As—Cu and As—Zn mixtures, and significant synergistic interactions for the Cu—Zn mixtures. Adding a second parameter b_{DL} to describe dose-level dependent deviations in the CA model provided the best model fits for As—Zn and Cu—Zn mixtures. In this case, the positive a value revealed an antagonistic interaction while the negative b_{DL} indicated that the magnitude of the antagonism was dose level dependent for As—Zn mixtures. For the Cu—Zn mixtures, the negative a value indicated that the interaction pattern at low dose levels was synergism, while the positive b_{DL} indicated a switch from synergism to antagonism at $1/b_{DL} = 1/1.10 = 0.9 * EC50$. Antagonistic effects of As—Cu and As—Ni on rice plants were also observed in soils (Tang and Miller, 1991). One latest study also showed the overall antagonistic interactions between As and Cu based on total metal concentrations in soil (Kilpi-Koski et al., 2020).

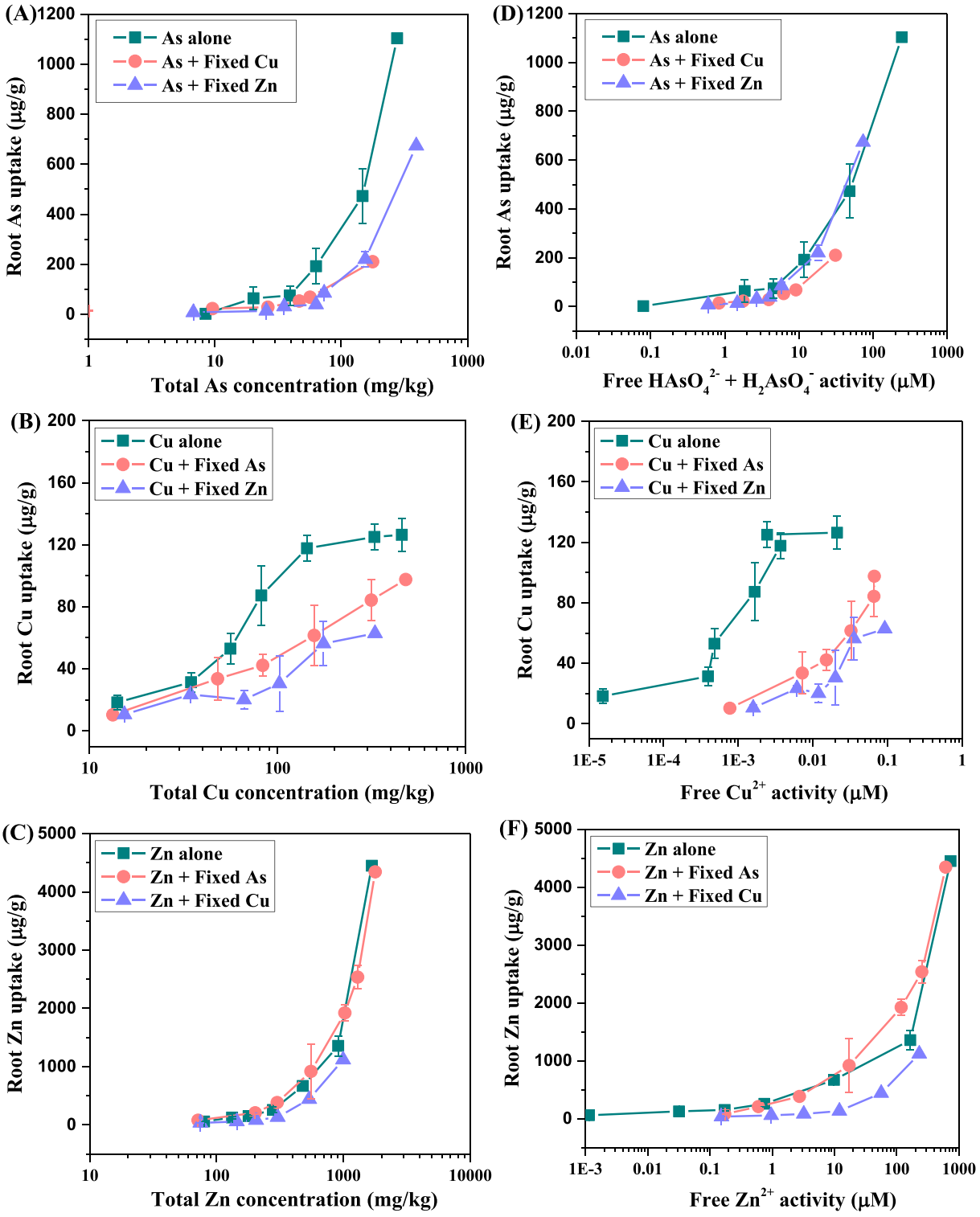

Fig. 2. Root uptake of As, Cu, and Zn in wheat when applied singly and in mixtures to LUFA 2.2 soil, and related to total exposure concentrations in soil (A–C) or free ion activity in the soil solution (D–F). In binary mixtures, the fixed concentrations of As, Cu, Zn were 60, 330, and 550 mg/kg, respectively. Solid lines schematically show the trends.

However, synergistic interactions of As—Cu mixtures were observed for acute toxicity with the worms (Wang et al., 2017). For As—Zn mixture, antagonistic interactions were often reported in soil (Das et al., 2005; Das et al., 2016; Guzman-Rangel et al., 2018). Antagonism based on the total metal concentrations in soil could be well interpreted by solubility interactions (Liu et al., 2007). For Cu- Zn mixture, Qiu et al. (2016) found synergistic effects on barley in medium/high-CEC soil but antagonism in low-CEC soil. Interestingly, both additive and synergistic effects on *Daphnia magna* were observed by Meyer et al. (2015). At this exposure level, the synergistic interactions in metal mixtures might be due to the components of the mixtures competing for sorption onto soil binding sites. Thus, one metal could be mobilized with the increasing total concentration of the other metal, thereby enhancing its bioavailability and toxicity (Qiu et al., 2011).

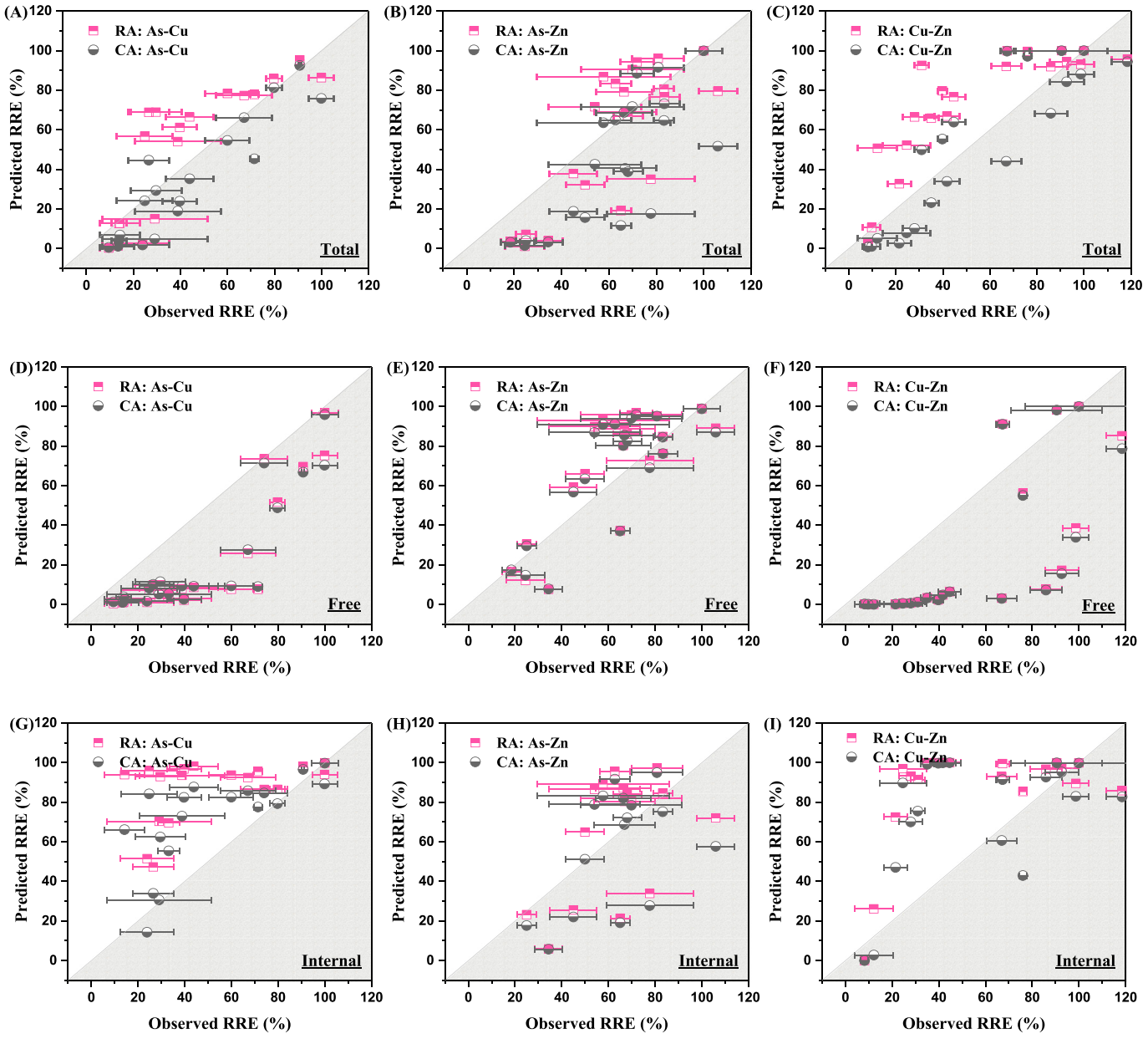

Fig. 3. The observed relative root elongation (RRE) of wheat following exposure to As—Cu (A, D, G), As—Zn (B, E, H) and Cu—Zn (C, F, I) versus values predicted by the reference models (CA and RA). Total metal concentration in soil (A, B, C), free ion activities in soil solution (D, E, F), and internal metal concentrations in plant roots (G, H, I) were used as the dose descriptor, respectively. The solid line represents the 1:1 line indicating additivity on the basis of the reference models. The grey area indicates synergism, while the white area indicates antagonism.

When we related the toxicity to {M}, adding the extra parameter a into the CA model to describe antagonism/synergism significantly improved the model fits for all three binary mixtures (Table 2). R^2 increased from 0.59 to 0.75 for the Cu—Zn mixtures with the estimated a value being positive, indicating antagonism (Tables 2 and S3). Adding parameters b_{DL} testing for dose level dependent deviations, improved the CA model fits significantly for the As—Cu and As—Zn mixtures. Thus, for the As—Cu mixtures, the interaction was antagonistic at low doses and switched to synergism at a dose of $1/b_{DL} = 1/0.1 = 10 * EC50$, and for the As—Zn mixtures, the interaction was synergistic at low doses and switched to antagonism at a dose of $1/b_{DL}$ of $1/0.7 = 1.4 * EC50$. The antagonistic interaction patterns were mostly expected for the mixtures containing As, because of the potential co-sorption or precipitation by the interaction between As and divalent metals (Das et al., 2005; Kader et al., 2017, 2018). Antagonistic interaction of Cu^{2+} and Zn^{2+} in soil solution was found in soil experiments with wheat due to their competition for binding sites (Qiu et al., 2016). Similarly, Cu^{2+}-Zn^{2+} antagonistic interactions in soil solution on barley growth were also observed (Versieren et al., 2014, 2017). Our results showed that the As and Zn uptake in the plant roots was dominated by their free ion activities. In other words, the free ions of As and Zn are the active species interacting with the plant root. On the basis of {M}, the less toxic Zn^{2+} might be replaced by the more toxic free As ions, leading to the synergistic interaction between As and Zn. Despite extensive publications dealing with metal mixtures, only few well documented studies report synergistic interactions of metal mixtures. The binary mixtures for which synergistic interaction were found include Cd + Zn, Cu + Zn, Cu + Cd and Cd + As to the shrimp *Penaeus setiferus*, the fish *Gobiocypris rarus* and the waterflea *Daphnia magna* (Cedergreen, 2014). The changes in metal speciation outside the organism, thereby influencing its bioavailability, might be a primary reason for the observed synergistic metal-metal interactions (Cedergreen, 2014).

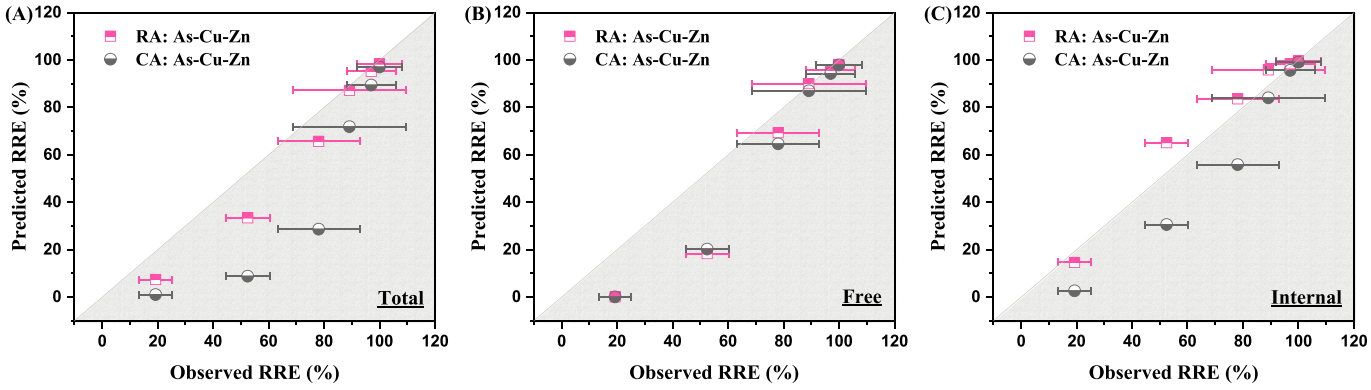

Fig. 4. The observed relative root elongation (RRE) of wheat following exposure to the ternary mixtures of As, Cu, and Zn versus values predicted by the reference model (CA and RA). Total metal concentrations in soil (A), free ion activities in soil solution (B), and internal metal concentrations in plant roots (C) were used as the dose descriptor. The solid line represents the 1:1 line indicating additivity on the basis of the reference models. The grey area indicates synergism, while the white area indicates antagonism.

When we related the toxicity to $[M]_{int}$, adding a positive parameter *a* and negative parameter b_{DL} into the CA model provided the best model fit, indicating that the deviation from additivity was mainly antagonistic and the magnitude of antagonism was dose level dependent for As—Zn mixtures. Antagonism in the As—Zn mixture seems fairly small considering the *a* value being close to zero and the *b* value being extremely low suggesting that the switch to synergism only occurs at very low levels compared to the EC50. Inclusion of negative parameters *a* decreased the *SS* significantly ($p\ (\chi^2) < 0.01$) for As—Cu and Cu—Zn mixtures, indicating the global interactions were mainly synergistic. Including a second parameter b_{DR}/b_{DL}, which tests for potential dose ratio or dose level dependent deviations, improved the CA model fits significantly for As—Cu and Cu—Zn mixtures. Thus, for the As—Cu mixtures, the interaction was synergistic when the mixture effect was caused mainly by Cu, and a decreased toxicity (antagonism) was occurred when the toxicity of the mixture was caused mainly by As. For the Cu—Zn mixtures, the interaction was synergistic at low doses and switched to antagonism at a dose of $1/b_{DL} = 1/0.82 = 1.2 * EC50$. The use of internal concentration as the dose descriptor takes into account the toxicokinetic of each mixture component (Ashauer and Escher, 2010). The competitive interaction between As and Zn partly occurred at the target level, causing the observed antagonistic interaction. Metal(loid) mixtures may put greater burdens on plant detoxification mechanisms, and thus result in the synergistic interactions. For example, Li et al. (2016) reported that a major detoxification mechanism of As in plants is binding with phytochelatins, sequestration to vacuoles and methylation. Phytochelatins or other antioxidant molecules could also detoxify the reactive oxygen species induced by As and Sb exposure (Feng et al., 2011). Therefore, it could be hypothesized that Cu and As (Zn) share the same detoxification mechanisms, leading to all the observed synergistic interactions of As—Cu, As—Zn, and Cu—Zn mixtures. Because there is almost no information for similar plant as the one selected for this study and for the metal combinations applied, it is difficult to compare our findings with those of others.

For the ternary As-Cu-Zn mixture, the CA and RA model predictions had almost similar precisions, with a good correlation between the observed RRE and the predicted values along the 1:1 line irrespective of the expressions of exposure. This suggests almost additive effects, except for the CA model using $[M]_{tot}$ as the dose descriptor. As for binary metal(loid) mixtures, for ternary mixtures more complicate interactions may occur. For example, noninteractive effects of the ternary mixture of Cu-Ni-Zn on algal growth were observed in two natural waters, whereas antagonistic interactions were found in one water with low-cationic competition situation (Van Regenmortel and De Schamphelaere, 2018). Additive effects on *Lemna minor* of co-exposure to Cd-Cu-Ni in alkaline solution (pH 8.3) using internal doses were also noted by Gopalapillai and Hale (2017). A ternary mixture of Cu-Cd-Pb produced antagonistic effects for the growth of the cucumber *Cucumis sativus* in soils (An et al., 2004). Based on total dissolved metal concentration, antagonistic toxicity of Cd-Cu-Zn mixtures to *Daphnia magna* was reported by Meyer et al. (2015). The toxicity of ternary Cd-Cu-Ni mixtures to *Daphnia magna* was antagonistic, additive, or synergistic, depending on the dose metrics used and the reference models applied. While the toxicity of ternary Cd-Ni-Zn mixtures was antagonistic or additive, depending on the dose metrics used but independent of the reference models applied (Traudt et al., 2017). In our study, no interaction of ternary As-Cu-Zn mixtures to wheat growth irrespective of the expressions of exposure was observed. This is likely

Table 2
Output parameters of the MIXTOX model and the identified interaction patterns for binary mixtures of As—Cu, As—Zn, and Cu—Zn with CA and RA as the reference model, respectively. Exposure was expressed as total soil metal concentration ($[M]_{tot}$, mg/kg), free metal ion activity in soil solution ({M}, μM), and internal metal concentration in the wheat root ($[M]_{int}$, μg/g), respectively.

Mixtures	Dose	CA-based						RA-based					
		a	b_{DR}/b_{DL}	$p\ (\chi^2)$	R^2	n	Pattern	*a*	b_{DR}/b_{DL}	$p\ (\chi^2)$	R^2	n	Pattern
As-Cu	$[M]_{tot}$	0.67	NA	0.00027	0.94	31	Antagonism	−5.8	8.3	0.0013	0.93	31	DR[b]
	{M}	14.8	0.10	0.0065	0.80	31	DL[a]	16.4	−22.6	0.00018	0.82	31	DR
	$[M]_{int}$	−3.48	0.56	0.0023	0.86	31	DR	−4.29	10.0	0.030	0.71	31	DR
As-Zn	$[M]_{tot}$	0.55	−1.88	0.0029	0.83	32	DL	0.0018	−2693	0.00052	0.79	32	DL
	{M}	−4.21	0.70	0.024	0.80	32	DL	−4.47	2.07	0.019	0.81	32	DL
	$[M]_{int}$	0.0042	−522	0.038	0.76	32	DL	0.0009	−4208	0.046	0.75	32	DL
Cu-Zn	$[M]_{tot}$	−1.29	1.10	0.017	0.90	33	DL	−4.60	NA	5.3E-9	0.87	33	Synergism
	{M}	49.9	NA	7.7E-11	0.75	33	Antagonism	50.6	NA	5.0E-9	0.76	33	Antagonism
	$[M]_{int}$	−2.85	0.82	0.099	0.45	33	DL	−17.7	21.9	0.014	0.52	33	DR

[a] DL: dose-level dependent.
[b] DR: dose-ratio dependent.

due to the use of only one soil type in our study, which greatly limited the influence of bioavailability on interactions and toxicity of all the tested mixtures (Qiu et al., 2011). It should be noted that the bioavailability of metal(loid)s could vary several orders of magnitude with the different soil properties, so further studies on the interaction patterns of metal(loid) mixtures are needed in more soil types.

4. Conclusions

The results of the present study shed light on the interaction mechanisms of metal(loid) mixtures (As, Cu, and Zn) in standard LUFA soil. The approach done in this research takes bioavailability into account which add new insights in standard phytotoxicity studies, that in many cases only use total metal content. The observed toxicity of the studied metal mixtures to wheat (*Triticum aestivum* L.) was quantified by different reference models (concentration addition and response addition) and related to total concentrations in soil, free ion activities in soil solution and internal concentrations in plant roots. Simple (antagonistic or synergistic) and more complex (dose ratio dependent or dose level dependent) interaction patterns of binary mixtures of As—Cu, As—Zn, and Cu—Zn occurred, depending on the dose metrics selected and the reference models used. For ternary mixtures (As-Cu-Zn), almost additive effects were observed irrespective of dose descriptors and reference models. Our results highlighted the importance of the reference models and the dose metrics in gaining reliable interaction mechanisms, which is helpful to accurately evaluate real scenarios where toxicity of metal(loid)s for soil-plant system should be studied.

CRediT authorship contribution statement

Bing Gong: Investigation, Writing - original draft. **Erkai He:** Conceptualization, Methodology, Writing - original draft, Funding acquisition. **Hao Qiu:** Conceptualization, Methodology, Writing - original draft, Funding acquisition. **Cornelis A.M. Van Gestel:** Writing - review & editing. **Ana Romero-Freire:** Writing - review & editing. **Ling Zhao:** Writing - review & editing. **Xinde Cao:** Writing - review & editing.

Declaration of competing interest

There is no competing interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.140926.

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